European Crop Protection

POSITION PAPER
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Request of the European Commission to the European Food Safety Authority (EFSA) for clarification and consideration of several aspects related to the assessment of genotoxicity

ECPA Input

Terms of reference and specific points to consider

ECPA general comments:

ECPA commend the Commission on recognising the concerns of industry over the strong divergence in opinion between some member states, EFSA and applicants regarding the assessment of genotoxicity databases.

- ECPA has identified that over the last several years the assessment of genotoxicity at EFSA has changed. Specifically there is clear evidence that EFSA now views genotoxicity as a set of independent endpoints and in direct contrast to the accepted practice of considering the outcome of these tests as indicative of the potential of chemicals to trigger a mode of action leading to carcinogenicity. For the databases developed on plant protection products this ignores the standard approach of considering genotoxicity as a part of the weight of the evidence including the outcome of carcinogenicity studies. This change of position by EFSA is not a consequence of any change in data requirement neither is it based on any recent scientific insights; moreover the changes appear to ECPA to be a deviation from EFSA's own position on using a weight of the evidence approach (1).
- The consequences of changes in approach by EFSA include an increase in the number of requests to either repeat and/or conduct additional studies in animals while not making any incremental contribution to human safety. Examples include an increase in vertebrate testing by repeating existing *in vivo* genotoxicity studies which do not have a proof of exposure of the target tissue or where the *in vivo* UDS assay is part of the dataset as a follow up to an *in vitro* gene mutation assay, while a larger body of evidence across all available studies including carcinogenicity would allow a confident conclusion on human safety.
- The current EFSA approach is contrary to the standard global regulatory approach which considers that genotoxicity is a mode of action causal to carcinogenicity. Any regulatory request triggering repeated and additional vertebrate testing outside of and in addition to standard requirements and approaches should be taken based on a cogent hypothesis related to protecting human health.

ECPA is encouraged to see the mandate to EFSA reflecting our specific concerns around:

- The challenge to the use of established assays (the *in vivo* UDS)
- The reluctance to accept reasonable scientific evidence of target organ exposure
- The deviation from EFSA's own position on using a weight of the evidence approach (1)

Based on these considerations, ECPA would urge EFSA to return to a more holistic assessment of all relevant data, i.e. all genotoxicity information *in vitro* and *in vivo*, carcinogenicity, and toxicokinetics as a part of a weight of the evidence evaluation.

In vivo Unscheduled DNA Synthesis (UDS) Assay

Key ECPA points:

- Data analysis has clearly shown that when the *in vivo* UDS has historically been used (for plant protection products) as a follow up to an *in vitro* positive mutagenicity signal, it has never failed to identify a chemical that was confirmed to be a genotoxin in carcinogenicity assays
- For existing datasets containing USD assays, a weight of the evidence approach should be taken to understanding genotoxic potential, including those data from carcinogenicity studies. Registrants should not be required to undertake additional and needless animal studies if the weight of evidence (including in higher tier studies) confirms a lack of genotoxicity resulting in carcinogenicity
- The mandate makes repeated reference to the Scientific Opinion (1) and that data generated since the publication of the opinion in 2011 should have followed its suggestions. However, EU1107/2009 listed the data requirements for active substances under EU No 544/2011 section 5.4.2. *In vivo* studies in somatic cells (10 June 2011). This states "If either of the *in vitro* gene mutation tests are positive, an in vivo test to investigate unscheduled DNA synthesis or a mouse spot test must be conducted". These data requirements were in place until the introduction of EU 283/2013. It is therefore misleading to propose in the Mandate that data generated after the publication of the Scientific Opinion and prior to the introduction of EU 283/2013 should have followed the opinion and NOT the specified data requirement as stated in the Mandate ". Hence the expectation should be that *in vivo* UDS data generated prior to EU 283/2013 should be accepted.
- The mandate reference (2) states that it was "concluded that the UDS Assay should not be used to follow up positive results in the *in vitro* gene mutation assay". However, this does not report the full opinion of the meeting. It is further reported "One expert maintained that the in vivo UDS assay may still be a valid and acceptable test under certain circumstances". Furthermore the mandate references the previous discussion on the utility of the UDS assay (3) without highlighting the outcome. The ECHA meeting noted, "It was concluded that it is a majority view that the UDS is adequate to detect some substances that induce gene mutations in the liver and that substance specific reasons can justify the use of the UDS", and further "the UDS might be equally adequate in some Cases, and substance-specific considerations should be taken into account".
- Considerations regarding the utility of the *in vivo* UDS assay have been essentially based on a single publication (i.e. Kirkland and Speit, 2008) (4) for which EFSA did not conduct its own independent review. The paper compares the performance of three *in vivo* genotoxicity assays (UDS, comet, and TGR) to identify rodent carcinogens that were previously negative in the *in vivo* micronucleus test viewing the data simply in a binary manner (positive or negative). The analysis did not take into account the mode of action (MoA)of the rodent carcinogens (genotoxic or nongenotoxic) used to compare the performance of the assays. There are several instances in the paper where rodent liver carcinogens produced a negative response in the UDS assay and thereby considered a failure to detect a positive response. In fact the negative UDS response was the correct call based on knowledge of the MoA and/or other pertinent information (e.g., phenobarbital – activation of CAR/PXR, carbon tetrachloride – excessive cytotoxicity; 1.3-dichloropropene – presence of a genotoxic stabilizer; di(2-ethylhexyl)phthalate and methyl clofenapate – peroxisome proliferation). Thus, the negative responses are entirely consistent with the nongenotoxic MoA that are known in these examples The role of cytotoxicity was also not taken into account in the induction of positive results in the

Comet assay for which data were cited principally from a single laboratory. In a recent publication (Speit et al., 2015 (5), an expert group concluded that cytotoxicity could be a confounder of comet results and it was recommended that multiple parameters be taken into account in order to determine if compound-specific toxicity may have influence the outcome of assay. Further, at the time of the analysis, the in vivo Comet and TGR assays did not yet have an accepted OECD test guideline in place (adopted 2014 and 2011, respectively). Most importantly, the analysis did not compare the three assays by looking across a common set of substances to evaluate the comparative performance. There are 10 rodent carcinogens in the Kirkland and Speit (2008) paper for which data from all three tests were available. The results of the UDS assay agreed with the other two tests in 7/10 (70%) of the instances (an equivocal response considered positive for this comparison) and 1 of 3 remaining substances was consistent with the TGR assay. Thus in 80% of the cases the UDS results matched the TGR and/or comet assay results. There were 16 chemicals for which data from both the transgenic and comet assays were available. In 81% of these cases (13/16) results of the two tests agreed. Therefore, the proportion of studies that resulted in comparable outcomes was the same for the two tests as it was for the three tests (~80%).

- Kirkland and Speit recognized the limitations of their analysis and stated in the paper that *"[a] full analysis of the performance of these assays across the wider database of rodent carcinogens (not just those that may be negative or equivocal in the micronucleus test) is on-going in order to see if these trends are maintained".* Thus, the conclusions and recommendations drawn from this paper are at best preliminary, and it was therefore premature to draw any significant conclusions regarding the comparative performance of these three in vivo genotoxicity tests.
- The proven utility of the *in vivo* UDS assay in the assessment of genotoxicity of an active ingredient should not be limited to those studies conducted prior to the change in data requirements. There will continue to be *in vivo* UDS studies conducted to meet other country regulatory requirements (for example India) in the future. Also the test is still listed under EU 283/2013 as one of two required follow up options to a gene mutation *in vitro* positive for the testing of micro-organism derived actives. ECPA believes that we should not be mandated to undertake further animal work by conducting a third *in vivo* study, such as a Comet or TGR assay when human health case the protected based on existing data or other established tests.

In vivo Unscheduled DNA Synthesis (UDS) Assay

Supporting information provided by ECPA

The data required for the registration of a plant protection product (PPP) active substance are extensive and therefore, these substances provide a unique data set across which it is possible to consider the use of the *in vivo* UDS assay in the identification of a genotoxic hazard and protection of human health. A survey of ECPA member companies was conducted on the use of the in vivo UDS assay. Participants submitted *in vitro* and *in vivo* genotoxicity results on registered PPP active substances where the genotoxicity assessment was supported by in vivo UDS data along with the outcome of the long-term carcinogenicity studies. Information on 54 data (53 registered actives and 1 awaiting registration confirmation) were considered. A total of 16 active substances had positive responses in the *in vitro* gene mutation assay (equivocal response considered positive). All 16 were negative in the *in vivo* UDS and micronucleus assays. Nine showed no treatment related neoplastic findings in the long-term carcinogenicity studies; therefore the *in vivo* UDS assay was in clear agreement with the cancer bioassay data. For the remaining 7 substances, tumorigenic responses were observed in the carcinogenicity studies; and based on mode of action understanding and/or weight of evidence analysis, all can be concluded as not being *in vivo* genotoxins. The *in vivo* UDS assay correctly predicted the lack of in vivo genotoxicity in all 16 of these cases, and this review supports the use of the *in vivo* UDS test as a continuing valuable assay in human safety assessments of PPP active substances.

Details of the analysis of PPP active substances are provided in the attached ECPA paper and accompanying flow chart.

UDS review short paper 3_March 2017

Evaluation of genotoxicity testing

Demonstration of target tissue exposure to the test substance

Key ECPA points:

- The *in vivo* bone marrow micronucleus assay is a widely used genotoxicity test. In the case of a negative result, the OECD test guideline for this assay states that it is required to demonstrate that the target tissue was exposed to the test article.
- OECD 474 Mammalian erythrocyte micronucleus assay (29 July 2016): "*Evidence of exposure of the bone marrow to a test substance may include a depression of the immature to mature erythrocyte ratio or measurement of the plasma or blood levels of the test substance. In case of intravenous administration, evidence of exposure is not needed. Alternatively, ADME data, obtained in an independent study using the same route and same species can be used to demonstrate bone marrow exposure.*"
- It is ECPA's understanding that the use of blood or plasma as a measure of bone marrow exposure is based on the ICH genotoxicity testing guidelines for pharmaceutical agents. ICH S2(R1), November 2011: "*Demonstration of in vivo exposure should be made by any of the following measurements: i. Cytotoxicity…….; ii. Exposure: Measurement of drug related material either in blood or plasma. The bone marrow is a well perfused tissue and*

levels of drug related materials in blood or plasma are generally similar to those observed in the bone marrow."

- Direct sampling of bone marrow is challenging due to accessibility and low sample mass from rodents, where the typical sample location is from the femurs.
- Blood collection provides an alternative less destructive method. The scientific basis for choosing blood is underpinned by an understanding of distribution within the mammalian body following administration of a compound.
- After administration of a test article the fraction absorbed into systemic circulation is distributed to the tissues. Anatomically, although the bone marrow represents about 2.3% of the body weight of rats, the mean regional blood blow to bone as a percent of cardiac output is estimated to be 12.2% indicating that this tissue is particularly well perfused (exposed).
- Distribution equilibrium between blood and tissue is generally reached more rapidly in richly vascularized, well perfused areas, such as bone marrow. After equilibrium, concentrations in tissues and in extracellular fluids are generally reflected by the blood concentration. Therefore, proof of exposure can be obtained by measurement of the compound of interest in blood.
- The advantages of using blood as a marker of exposure include reduced animal usage, ease of access and quantitative collection and availability of relatively straightforward high sensitive (e.g., LC/MS) analytical methods for test substance/material analysis.

Demonstration of target tissue exposure to the test substance

Supporting information provided by ECPA:

ECPA Proof of Exposure for Micron

The use of data in a weight of the evidence approach to conclude on the genotoxic potential of active substances and their metabolites and the setting of health-based reference values for use in human health risk assessment

Key ECPA points:

The use of a weight of evidence approach, including consideration of the long-term carcinogenicity studies, QSAR, ADME, and other relevant information, should be consistent with the existing recommendations of the 2011 EFSA scientific opinion on genotoxicity testing strategies (1). The following excerpts from the opinion are particularly relevant to EFSA's sated approach:

- **Page 33:** "*Since in vivo tests take into account absorption, distribution and excretion* [ADME] *(this is not the case for in vitro tests), they are considered as potentially relevant to human exposure. In addition, metabolism is likely to be more relevant in vivo compared with systems normally used in vitro. When in vivo and in vitro results are not consistent, then the differences should be clarified on a case-by-case basis.*"
- **Page 35:** "*In cases where limited or no test data are available, the (Q)SAR approach could be useful in a weight-of-evidence approach that includes information from all available sources (e.g., read-across and experimental data).*"
- **Page 43:** "*The Scientific Committee recognizes that EFSA will continue to receive datasets that differ from the testing strategy recommended in this opinion. Such databases* ***should be considered on a case-by-case basis*** [emphasis added]. *Provided the three critical endpoints (i.e., gene mutation, structural and numerical chromosome aberration) have been adequately investigated, such datasets may be considered acceptable. The Scientific Committee recognizes that in other cases where there is a heterogeneous dataset, EFSA has to rely on a weight-of-evidence approach.*"
- **Page 44:** "*The Scientific Committee recommends a* ***documented weight-of-evidence approach to the evaluation and interpretation of genotoxicity data*** [emphasis added]. *Such an approach should not only consider the quality and reliability of the data on genotoxicity itself, but also take into account other relevant data that may be available, such as physico-chemical characteristics, structure -activity relationships (including structural alerts for genotoxicity and read-across from structurally related substances), ADME, and the outcomes of any repeat-dose toxicity and carcinogenicity studies. The use of all available relevant data is critical to reaching a sound conclusion on genotoxic potential as well as assisting in the design of genotoxicity studies and decision-making on the strategy for follow-up of positive or equivocal results from testing in the basic battery.*"

Although it is recognised that non-carcinogenic disease patterns can be linked to the detection of DNA damage in the scientific literature, it is only an assumption on the part of EFSA that this link is causal with genotoxicity being the molecular initiating event (i.e. genotoxicity leads to the disease patterns to which they get linked). A more accepted scientific interpretation of the data is that the DNA damage detected in diseases flagged by EFSA (chronic inflammatory diseases, cardiovascular diseases etc) is more likely a secondary consequence to persistent endogenous inflammatory events.

Based on these considerations, ECPA would urge EFSA to return to a more holistic assessment of all relevant data, i.e. all genotoxicity information *in vitro* and *in vivo*, carcinogenicity, and toxicokinetics as a part of a weight of the evidence evaluation.

References

(1) EFSA Scientific Committee; Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379. [68 pp.] doi:10.2903/j.efsa.2011.2379. Available online: www.efsa.europa.eu/efsajournal
2) Technical report on the outcome of the pesticides peer review meeting on general recurring issues in mammalian toxicology. EFSA supporting publication 2016:EN-1074. 24 pp.
(3) Technical Discussion Session on the Scientific Adequacy of in vivo Mutagenicity Assays, the Transgenic Rodent Gene Mutation Assay and the Unscheduled DNA Synthesis Assay. Report Helsinki, 4 October 2012.
4) Kirkland D., Speit G. (2008) Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. III. Appropriate follow-up testing in vivo. Mutat. Res. 654:114-32.
(5) Speit G., et al., (2015) Critical issues with the in vivo comet assay: A reportof the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT). Mutat. Res. Genet. Toxicol. Environ. Mutagen. 783:6-12.